Carboxy groups as essential residues in β -lactamases

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1. β -Lactamases are divided into classes A, B and C on the basis of their amino acid sequences. 2. β -Lactamases were incubated at pH 4.0 with the carboxy-group reagent 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide plus a coloured nucleophile and the extents of inactivation and nucleophile incorporation were monitored. 3. Two class A enzymes (from *Bacillus cereus* and *Bacillus licheniformis*) and two class C enzymes (from *Enterobacter cloacae* P99 and *Pseudomonas aeruginosa*) were examined. 4. All four enzymes were inactivated, with total inactivation corresponding to the incorporation of approx. 2–3 mol of nucleophile/mol of enzyme. 5. In the case of β -lactamase I from *Bacillus cereus*, some 53% of the incorporated nucleophile was located on glutamic acid-168 in the amino acid sequence.

INTRODUCTION

β -Lactamases are clinically important enzymes because of the part they play in resistance to antibiotics. They are a remarkably diverse group of enzymes when their substrate specificity is considered, but have been divided into three classes (A, B and C) on the basis of their amino acid sequences (Ambler, 1980; Jaurin & Grunström, 1981). Mechanistically, the β -lactamases are either 'serine enzymes' or 'zinc enzymes' (see, e.g., Bicknell & Waley, 1985). The chemical modification of carboxy groups in β -lactamase I (Waley, 1975) and β -lactamase II (Little *et al.*, 1986) (both enzymes are from *Bacillus cereus* and are respectively a serine enzyme and a zinc enzyme) causes inactivation. The present paper on the one hand extends the study of the effect of carboxy-group modification to other serine β -lactamases, and on the other hand shows that in β -lactamase I (*Bacillus cereus*) glutamic acid-168 is a major site of labelling during the inactivation process.

MATERIALS AND METHODS

Materials

β -Lactamase I was purified from the culture supernatant of *B. cereus* 569/H/9 as described (Davies *et al.*, 1974). The β -lactamase from a mutant of *Pseudomonas aeruginosa* 18S (Sabath *et al.*, 1965; Flett *et al.*, 1976; Berks *et al.*, 1982) was purified by affinity chromatography (Courtwright & Waley, 1984), as was the P99 β -lactamase (Ross, 1975) from *Enterobacter cloacae*. The β -lactamase from *B. licheniformis* was a gift from J.-M. Frère (Université de Liège, Liège, Belgium). EDC was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and Dnp-ethylenediamine hydrochloride from Calbiochem, Lucerne, Switzerland.

Assay of enzyme activity

Enzyme activity was measured at 30 °C at pH 6.0 by continuous titration as described by Davies *et al.* (1974).

Inactivation conditions

Unless otherwise stated, enzyme (1–2 mg/ml) was incubated in 0.5 M-NaCl/5 mM-Dnp-ethylenediamine. The reaction mixture was maintained at pH 4.0 at 22–24 °C in a pH-stat. When the extent of incorporation of nucleophile into the enzyme was to be measured, the enzyme was incubated with EDC and Dnp-ethylenediamine for 45 min and then dialysed at 4 °C against three changes of 0.5 M-NaCl/10 mM-sodium acetate buffer, pH 6.0. The modified enzyme was centrifuged (5000 *g* for 5 min) to remove insoluble material and then the nucleophile concentration was measured at 360 nm by using $\epsilon = 15000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Huc *et al.*, 1975). Enzyme concentrations were calculated from measurements of A_{280} by using $\epsilon = 26000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for β -lactamase I (Davies, 1973), $\epsilon = 73000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for the P99 β -lactamase (Joris *et al.*, 1985), $\epsilon = 70000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for the *Pseudomonas* β -lactamase (McPhail & Furth, 1973) and $\epsilon = 28000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (calculated from the A_{280} of a solution containing a known weight of freeze-dried enzyme) for the *Bacillus licheniformis* enzyme. The A_{280} of solutions of enzyme containing incorporated nucleophile was corrected for the A_{280} of the latter according to the equation:

$$A_{280}(\text{enzyme}) = A_{280}(\text{complex}) - \frac{A_{360}(\text{complex})}{3}$$

where $A_{280}(\text{enzyme})$ is the true A_{280} from the protein, and $A_{280}(\text{complex})$ and $A_{360}(\text{complex})$ refer to the absorbances of the modified enzyme.

For the preparation of labelled peptides, about 20 mg of β -lactamase I was inactivated with EDC and Dnp-ethylenediamine and dialysed against 0.5 M-NaCl/10 mM-sodium acetate buffer, pH 6.0, and then against 50 mM-NH₄HCO₃ and finally freeze-dried. The residue was taken up in 1 ml of 1% (w/v) NH₄HCO₃ and to this was added 1.6 mg of trypsin in 0.16 ml of 1 mM-HCl. The mixture was agitated at 37 °C for 6 h and then subjected to gel filtration.

Abbreviations used: EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide [1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide]; Dnp, 2,4-dinitrophenyl.

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H.p.l.c.

Reverse-phase h.p.l.c. was performed in a Lichrosorb RP (5 μ m particle size) column (4.6 mm $\times$ 250 mm). The elution medium was 0.1% trifluoroacetic acid (solvent A) and acetonitrile/propanol/0.1% trifluoroacetic acid (4:2:1, by vol.) (solvent B). A linear gradient of solvent B in solvent A was developed over 60 min.

Peptide analysis

For amino acid analyses, performic acid-oxidized samples were hydrolysed in 6 M-HCl at 110 °C for 18 h. Amino acid composition was determined with a Biochrom 4101 analyser (LKB, Bromma, Sweden). *N*-Terminal peptide residues were identified by the dansyl procedure of Bruton & Hartley (1970). Automatic liquid-phase sequencing of peptides was performed in a Beckman 890C Sequenator and the amino acid residues were identified by h.p.l.c. (Campbell *et al.*, 1981).

RESULTS

Inactivation of four different β -lactamases by carbodi-imide plus nucleophile

When β -lactamases enzymes from *B. cereus*, *B. licheniformis*, *E. cloacae* P99 and *Ps. aeruginosa* were incubated with the water-soluble carbodi-imide EDC together with the coloured nucleophile Dnp-ethylenediamine at room temperature and pH 4.0, a rapid inactivation process occurred with all four enzymes, the reaction ceasing within some 30–40 min. By using different amounts of the carbodi-imide various extents of inactivation could be produced, and when free nucleophile was removed by extensive dialysis the extents of inactivation and the corresponding extents of incorporation of nucleophile into each of the four enzymes could be measured. The results are shown in Fig. 1. For the enzymes from *B. cereus*, *B. licheniformis* and *E. cloacae* a linear relationship was observed, with total inactivation corresponding to the incorporation of approx. 2.1, 3.0 and 2.4 mol of nucleophile/mol of enzyme respectively. In the case of the *Ps. aeruginosa* enzyme it seemed that 1 mol of nucleophile could be incorporated/mol of enzyme without any change occurring in the catalytic activity. Incorporation of a further approx. 1.8 mol of nucleophile/mol seemed then to result in total inactivation. The extent of nucleophile incorporation was not changed by exposure of any of the modified enzymes to 0.2 M-hydroxylamine for 24 h at room temperature at pH 7.0. This would suggest that no tyrosine modification had occurred (Carraway & Koshland, 1972).

With strongly inactivated samples of the enzymes we normally observed the formation of small amounts of insoluble heavily modified material. This was removed by centrifugation before measurements of residual enzyme activity and extent of nucleotide incorporation. The activity and nucleophile incorporation data shown in Fig. 1 have been corrected for this loss of protein and therefore refer to enzyme remaining in the soluble form after treatment.

Labelled peptides from β -lactamase I (*B. cereus*)

The extent of carboxy-group modification for total inactivation was significantly smaller in the case of the *B. cereus* and *E. cloacae* enzymes than with the other two β -lactamases examined. Since the complete amino acid

sequence of the *B. cereus* enzyme is known, whereas only partial sequencing data on the *E. cloacae* enzyme have been published (Joris *et al.*, 1984, 1985), the former enzyme was selected for a study of the sites of labelling by the carbodi-imide plus nucleophile. Samples of β -lactamase I (*B. cereus*) containing 0.8 and 1.7 mol of label/mol of enzyme (residual catalytic activities 65 and 15% respectively) were digested with trypsin and subjected to gel filtration in 10% (v/v) acetic acid. Four discernible peaks of labelled material were obtained from each enzyme sample (Fig. 2). Fractions were pooled as indicated in the Figure. The four pools of labelled material were dried down and further analysed and fractionated by h.p.l.c. Gel-filtration pools I, III and IV yielded in each case one totally dominating peak of labelled material, whereas pool II yielded a major peak comprising about 40% of the label in that pool with the remaining 60% being smeared out over several small poorly resolved peaks. Pooling and re-running this latter material in h.p.l.c. simply confirmed that it comprised a large number of components all present in small amounts only (results not shown). The five fractions comprising the peak of labelled material from gel-filtration pool I were dried down and resubmitted to h.p.l.c. but with elution with a narrower gradient [30–80% (v/v) solvent B in solvent A; see the Materials and methods section]. Again a single peak of labelled material was observed (results not shown). The peak fractions (containing about 70% of the amount of label loaded) were pooled and dried down before being analysed for amino acid composition and sequence and *N*-terminal determination. The results are shown in Fig. 3 and Table 1. A single highly purified peptide had been isolated. The first ten residues of this peptide had an amino acid sequence identical with that of residues 165–174 in the native enzyme. The labelled residue Xaa is therefore the glutamic acid-168 residue. In some samples of enzyme analysed, a minor amount of labelling (less than 10%) was also observed on the glutamic acid-171 residue.

DISCUSSION

Under relatively mild conditions of temperature, incubation time, pH and reagent concentration four different β -lactamases were inactivated by a carboxy-group-modifying reagent. None of these enzymes is a thiol enzyme, so it is unlikely that the inactivation involved the modification of essential thiol groups; this point is significant, since thiol groups and carboxy groups if sterically available are generally equally readily modified by carbodi-imides (Carraway & Koshland, 1972). Furthermore, tyrosine modification was also shown to be unlikely (see above). It would therefore seem probable that the inactivation of these four enzymes is attributable to the modification of carboxy groups. The stoichiometry of inactivation as shown in Fig. 1 indicates a linear relationship between the extents of inactivation and of label incorporation, though in the case of one enzyme there was a lag phase in this process before inactivation. For total inactivation only relatively modest extents of enzyme modification (two or three residues of label incorporated/molecule of enzyme inactivated) were required. This suggests a fairly selective and specific mechanism of inactivation rather than one involving a general denaturation of the enzymes as a

Fig. 1. Stoichiometry of inactivation of four different β -lactamases by carbodi-imide plus nucleophile

Enzymes were incubated in 3 ml portions with Dnp-ethylenediamine plus various concentrations of carbodi-imide (1–15 mM). The residual enzyme activities and extents of label incorporation were measured. For further details see the Materials and methods section. The β -lactamases studied were from *B. cereus* (a), *E. cloacae* (b), *B. licheniformis* (c) and *Ps. aeruginosa* (d). The insets show the inactivation data for each enzyme plotted in the form $a^{1/4}$ versus m , where a is the fractional residual catalytic activity and m is the number of Dnp groups incorporated. Values of i used were 1 (●) and 2 (○).

result of extensive chemical modification. Various methods exist for further analysis of inactivation stoichiometry data (see Ray & Koshland, 1961; Tsou, 1962; Horiike *et al.*, 1984). Analysis of the data by the simplest method of Tsou (1962) (Fig. 1 insets) indicated that for the β -lactamases from *B. cereus* and *E. cloacae* the results fitted well with the existence of only one essential carboxy group in each enzyme. The data obtained with the *B. licheniformis* and *Ps. aeruginosa* enzymes were not of adequate quality to permit the use of this method to distinguish between the presence of one or of two essential carboxy groups. However, the presence in the *Ps. aeruginosa* enzyme of a single extremely reactive non-essential residue is also apparent in the data replots (Fig. 1d inset). When our previous

results (Little *et al.*, 1986) are included, it can be seen that β -lactamases of all three classes are readily inactivated by exposure to the particular water-soluble carbodi-imide used and that it is therefore possible that active-site carboxy groups are a common feature of β -lactamases. What precise function, if any, such 'essential' carboxy groups might fulfil cannot be established until the active sites of these enzymes are characterized far better.

An attempt was made to analyse more closely the sites in β -lactamase I (*B. cereus*) that were labelled during enzyme inactivation by the carbodi-imide plus nucleophile. From a tryptic digest of the labelled enzyme four fractions of labelled material were isolated by gel filtration. The fraction containing the greatest amount of label was further purified by h.p.l.c. and found to

Fig. 2. Separation of tryptic peptides from modified β -lactamase I (*B. cereus*) by gel filtration

A tryptic digest of 20 mg of modified enzyme (1.1 ml) containing 1.7 mol of Dnp group/mol of enzyme was applied to a column (0.45 mm $\times$ 140 cm) of Sephadex G-25 and eluted with 10% (w/v) acetic acid. Fractions (approx. 1 ml) were collected and the A_{280} (not shown) and A_{360} were measured. Fractions were pooled as shown (I–IV).

Phe-Glu-Thr-Xaa-Leu-Asn-Glu-Ala-Ile-Pro-Gly-Asp-Ile-Arg

Fig. 3. Sequence of labelled peptide from modified β -lactamase I (*B. cereus*)

The unidentified residue, Xaa, was strongly yellow-coloured.

Table 1. Composition of labelled tryptic peptide from modified β -lactamase I (*B. cereus*)

Component	Composition (mol/mol of peptide)	
	Observed	Expected*
Aspartic acid	2.0	2
Threonine	0.8	1
Serine	0.5	0
Glutamic acid	3.1	3
Proline	0.95	1
Glycine	1.4	1
Alanine	1.4	1
Isoleucine	1.6	2
Leucine	1.3	1
Phenylalanine	0.9	1
Lysine	0.4	0
Arginine	0.5	1
Dnp	1.1	1
N-Terminus . . .	Phenylalanine	Phenylalanine

* Expected for residues 143–157.

contain a single labelled peptide corresponding to residues 164–174 in the intact enzyme, with the label residing on the glutamic acid-168 residue. When the distribution of label among the peaks of labelled

Table 2. Distribution of label among peptides from pooled fractions from gel filtration of trypsin-digested β -lactamase I (*B. cereus*)

Pools I, II, III and IV of labelled peptides (see Fig. 2) were further fractionated by h.p.l.c. with a linear gradient of solvent B in solvent A (see the Materials and methods section). Each pool yielded one major peak of yellow material centred around fraction no. 30 (fraction volume 0.75 ml) for pools I and II and around fraction no. 27 for pools III and IV.

Gel-filtration pool	Label content (% of all label)	No. of major labelled peaks found in h.p.l.c.
I	53	1
II	0 (8 + 12)	1 (8%) + many minor (12%)
III	17	1
IV	9	1

material seen in gel filtration (Fig. 2) is considered, it can be shown that this particular peptide accounts for some 53% of the total incorporated nucleophile (Table 2). Fraction II contained about 20% of the incorporated label, but on further analysis it was found that of this 20% some 12% was spread amongst many minor components and that the remaining 8% occurred in a single sharp peak in h.p.l.c. at the same point in the elution gradient as the labelled peptide from fraction I appeared (Table 2). Fraction III yielded in h.p.l.c. a single peak of labelled material representing about 17% of the incorporated nucleophile, with a further 9% being observed in a single peak in h.p.l.c. from fraction IV. Within experimental error, the same distributions of label were noted in samples of labelled enzyme containing 0.8 and 1.7 mol of label/mol of enzyme, i.e. 45% and 85% enzyme inactivation respectively. Taken together these results are consistent with the view that during the inactivation of β -lactamase I (*B. cereus*) by the carbodi-imide plus nucleophile essentially one major and two or three minor sites of labelling exist. If the major peak of labelled material from fraction II represents the same peptide as that isolated and sequenced from fraction I, then the minor components contain too little label (total about 34% of that incorporated) to account for the inactivation. A small amount of double labelling was observed in the sequenced peptide (minor labelling on the glutamic acid-171 residue), but the overwhelming proportion of the label therein occurred at the glutamic acid-168 residue. Total inactivation of this enzyme is associated with the binding of approx. 2 mol of nucleophile/mol of enzyme. Glutamic acid-168 would seem to be an essential residue, but, in view of the above stoichiometry, there may be another essential carboxy group in the enzyme. However, further analysis of the data for β -lactamase I (*B. cereus*) inactivation suggested the existence of a single essential carboxy group. We therefore consider it probable that it is the conversion of glutamic acid-168 into the amide that renders the enzyme inactive. Although glutamic acid-168 is conserved in the class A β -lactamases cited by Ambler (1980), it is replaced by lysine in the sequence of the β -lactamase from *Rhodopseudomonas capsulata* (J. Campbell &

R. P. Ambler, personal communication). Moreover, glutamic acid-168 has been replaced by aspartic acid by mutagenesis without appreciable effect on activity (P. J. Madgwick & S. G. Waley, unpublished work). Thus glutamic acid-168 is not an essential residue. However, glutamic acid-166 is a conserved residue; thus the conversion of glutamic acid-168 into a bulky residue might destroy catalytic activity by influencing a nearby catalytically functional residue.

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